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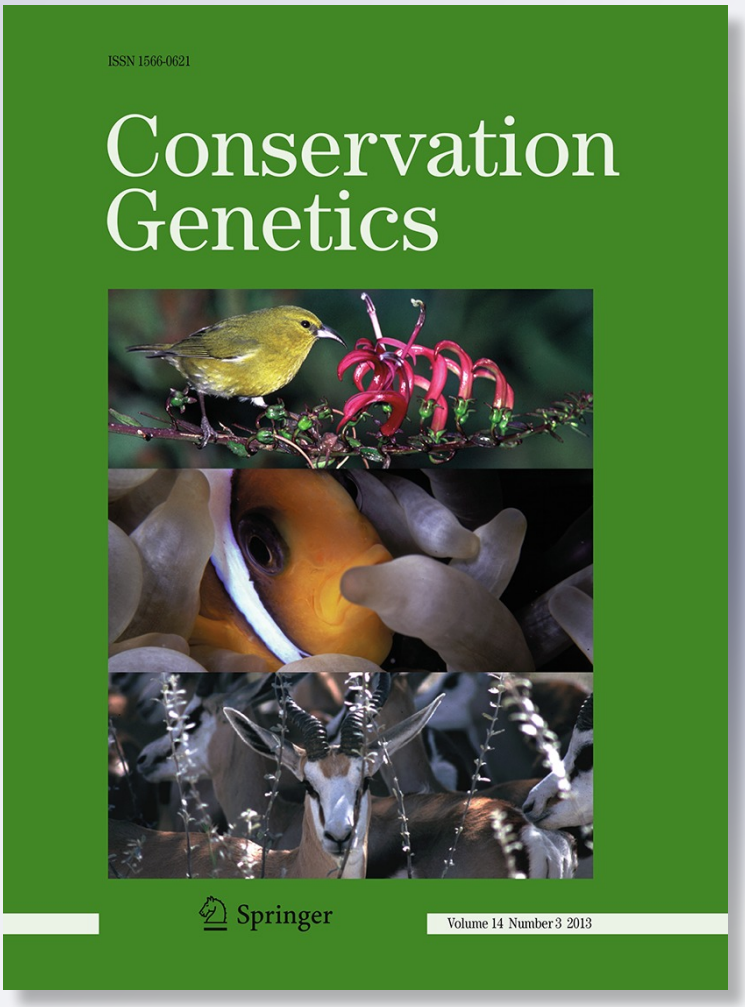

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RESEARCH ARTICLE

Population stock structure of leatherback turtles (*Dermochelys coriacea*) in the Atlantic revealed using mtDNA and microsatellite markers

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Abstract This study presents a comprehensive genetic analysis of stock structure for leatherback turtles (*Dermochelys coriacea*), combining 17 microsatellite loci and 763 bp of the mtDNA control region. Recently discovered eastern Atlantic nesting populations of this critically endangered species were absent in a previous survey that found little ocean-wide mtDNA variation. We added rookeries in West Africa and Brazil and generated longer sequences for previously analyzed samples. A total of 1,417 individuals were sampled from nine nesting sites in the Atlantic and SW Indian Ocean. We detected additional mtDNA variation with the longer sequences, identifying ten polymorphic sites that resolved a total of ten haplotypes, including three new variants of haplotypes previously described by shorter sequences. Population differentiation was substantial between all but two adjacent rookery pairs, and F_{ST} values ranged from 0.034 to 0.676 and 0.004 to 0.205 for mtDNA and microsatellite data respectively, suggesting that male-mediated gene flow is not as widespread as previously assumed. We detected weak ($F_{ST} = 0.008$ and 0.006) but significant differentiation with microsatellites between the two population pairs that were indistinguishable with mtDNA data. POWSIM analysis showed that our mtDNA marker had very low statistical power to detect weak structure ($F_{ST} < 0.005$), while our

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microsatellite marker array had high power. We conclude that the weak differentiation detected with microsatellites reflects a fine scale level of demographic independence that warrants recognition, and that all nine of the nesting colonies should be considered as demographically independent populations for conservation. Our findings illustrate the importance of evaluating the power of specific genetic markers to detect structure in order to correctly identify the appropriate population units to conserve.

Keywords Sea turtle · *Dermochelys coriacea* · Conservation genetics · Mitochondrial DNA · Demographically independent populations · Management · Recovery plan · Microsatellites

Introduction

In recent years, molecular techniques have played a significant role in the conservation and management of a variety of large marine vertebrates such as beluga whales (*Delphinapterus leucas*), harbor porpoises (*Phocoena phocoena*) (O'Corry-Crowe et al. 1997; Chivers et al. 2002), sperm whales (*Physeter macrocephalus*) (Mesnick et al. 2011) and all the marine turtle species (Jensen et al. in press). Despite the capacity for wide dispersal in the marine environment, many marine species exhibit population genetic structuring. For example, endangered Steller sea lion (*Eumetopias jubatus*) populations have strong matrilineal divisions as determined using mtDNA and little evidence of genetic structure with nuclear microsatellite markers (O'Corry-Crowe et al. 2006). This is interpreted to indicate extensive paternal gene flow in a continuously distributed population (Hoffman et al. 2006). Additionally, a molecular study of sympatric killer whales (*Orcinus orca*) revealed genetically distinct populations coexisting within a local geographical range (Hoelzel and Dover 1991). Marine turtles also show strong mtDNA matrilineal divisions shaped by natal homing to breeding sites, and often exhibit greater corresponding nuclear (microsatellite) homogeneity, a pattern generally attributed to male-mediated gene flow (Bowen et al. 2005; Carreras et al. 2007).

Several concepts have been used to describe population units for conservation that are relevant to ecological and evolutionary timescales below the species level. Evolutionarily significant units (ESU) and distinct population segments (DPS) are broader units, generally shaped by processes on evolutionary timescales that are defined by global divisions in the patterns of genetic variation and are relatively easy to detect with standard genetic markers, such as mtDNA, when they exist (Taylor et al. 2010). Finer scale structuring within ESUs or DPSs, often shaped by environmental or behavioral processes on ecological timescales, comprises groups of animals that are demographically independent. The level of connectivity among these smaller scale groups, typically defined as management units (MUs), or demographically independent populations (DIPs), is important to characterizing the overall population structure and vulnerability to threats. MUs are defined by significant divergence of nuclear or mtDNA allele frequencies (Moritz 1994) although it is harder to detect low levels of differentiation that characterize DIPs (Taylor et al. 2010) with the genetic markers that are typically available, and failure to detect demographic independence when it exists may lead to inappropriate management policy (Taylor and Dizon 1999).

Setting appropriate conservation priorities is important for sea turtles since all species have several populations which are threatened with extinction (Wallace et al. 2011). In particular, the leatherback, *Dermochelys coriacea*, largest of all species of marine turtles, is distributed worldwide in tropical and subtropical waters. The species is considered critically endangered worldwide by the International Union for Conservation of Nature (IUCN 2009), and despite the efforts of conservationists and scientists over the past few decades, some leatherback turtle rookeries are still experiencing population declines, particularly in the Pacific (Liew 2011; Sarti Martinez and Barragan 2011; Tapilatu et al. 2013). Within the Atlantic basin, leatherbacks nest widely, and many Caribbean populations are considered to be increasing (Dutton et al. 2005; Turtle Expert Working Group 2007; Stewart et al. 2011), although in the southwestern Atlantic only small scattered nesting remains in northern Brazil. In the southeastern Atlantic, large nesting populations have been discovered in Gabon (Witt et al. 2009). However these appear to be under pressure from multiple threats along the coast of West Africa (Witt et al. 2011). There is also concern that despite over 40 years of protection, the leatherback population nesting in South Africa remains depleted (Ronel Nel personnel communication). The leatherback's broad oceanic distribution and highly migratory nature makes studying its movements and life history difficult. Tag-return data, along with mtDNA control region haplotype frequencies, have been used successfully to support the natal homing hypothesis by indicating that there is restricted dispersal in female leatherback turtle nesting sites (Dutton et al. 1999). MtDNA techniques, which characterize maternal lineages within species, have been useful for distinguishing major rookeries, or MUs, over broad geographic scales (Avise 1998), but may not provide sufficient fine-scale resolution when the amount of haplotype frequency overlap between nearby rookeries becomes more widespread (Velez-Zuazo et al. 2008; LeRoux et al. 2012). This is particularly the case for leatherbacks, which are characterized by a low level of mtDNA variation (Dutton et al. 1999). Earlier studies using 496 bp mtDNA sequences were useful in revealing stock structure and

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phylogeography of leatherbacks on a global scale (Dutton et al. 1999, 2007). However, because one ubiquitous mtDNA haplotype is present in all Atlantic leatherback rookeries, the estimation of stock composition of regional foraging populations, as well as the stock assignment of incidentally caught or stranded leatherbacks has been problematic. The lack of mtDNA variation in leatherbacks and inadequate sampling of key nesting sites has also made it impossible to accurately characterize fine-scale stock structure within the Atlantic and Caribbean. Recent studies using longer (>700 bp) sequences have uncovered additional variation in mtDNA control region (CR) that has improved the ability to detect population structure in loggerhead (Monzón-Argüello et al. 2010; Shamblin et al. 2012) and hawksbill turtles (LeRoux et al. 2012). Furthermore, there is an increasing recognition for the need to incorporate nuclear markers into population genetic studies, since the single organelle data from mtDNA sequences only reflects variation among female lineages (Bowen et al. 2005; Carreras et al. 2007). Because little is known about the movements and reproductive behavior of male leatherback turtles, the use of nuclear DNA markers (microsatellites) may help define population structure further and indicate if gene flow is male-mediated. Furthermore, key Atlantic rookeries have not been surveyed in earlier studies (Dutton et al. 1999; Vargas et al. 2008).

Here we reanalyze samples from previous studies (Dutton et al. 1999) using new mtDNA primers that provide longer sequences to determine whether new genetic variation can be uncovered to improve the detection of population structuring and to describe the demographic history of Atlantic leatherbacks. In addition, we used 17 polymorphic microsatellite loci to supplement the population stock structure defined with mtDNA analysis. We also added samples from rookeries in West Africa, Brazil, and larger sample sizes from rapidly expanding rookeries in Florida and St. Croix in the US Virgin Islands. Defining stock boundaries and evolutionarily significant units for this species will be useful for setting conservation priorities (Karl and Bowen 1999). In addition, the identification of stocks will allow for other management needs such as identifying source rookeries for leatherbacks incidentally caught in high-seas fisheries, defining foraging areas for various nesting rookeries, and mixed-stock analysis (MSA) of foraging populations. We assess whether the leatherback's potential for dispersal over long distances is translated into broad gene flow across its range or if microsatellites can detect population subdivision. This study provides a solid baseline for future MSA, as well as many other analyses relevant to management and recovery plans for the species.

Methods

Sample collection

Blood or skin samples representing nesting populations distributed throughout the Atlantic and adjacent Indian Ocean were collected from nesting leatherbacks or salvaged from dead hatchlings using protocols described in Dutton (1996) and Dutton et al. (1999) (Fig. 1, see Online Resource 1 for detailed description of nesting populations and sampling protocols).

Laboratory procedures

We used standard manufacturer protocols to extract total genomic DNA using the following methods: phenol/

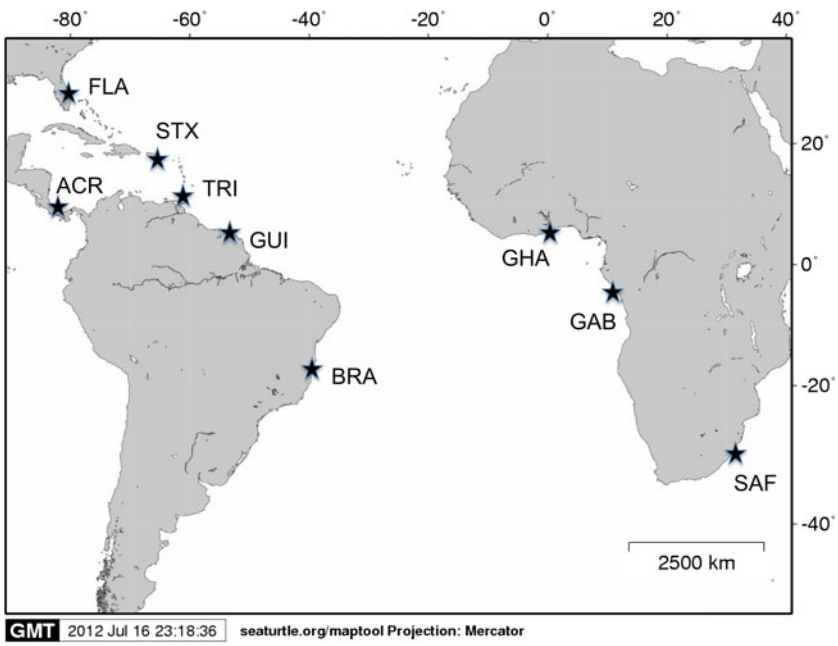

Fig. 1 Locations of sampled leatherback nesting sites in the Atlantic and Indian Ocean, including Brazil (*BRA*), Atlantic Costa Rica (*ACR*), French Guiana and Suriname (*GUI*), Gabon (*GAB*), Ghana (*GHA*), Trinidad (*TRI*), Florida (*FLA*) in the United States, St. Croix (*STX*) in the U.S. Virgin Islands and South Africa (*SAF*). Detailed description of nesting sites are given in Online Resource 1

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chloroform (modified from Sambrook et al. 1989), sodium chloride extraction (modified from Miller et al. 1988), X-tractor Gene robot, or modified DNEasy® Qiagen extraction kit (Qiagen, Valencia, CA, USA).

Mitochondrial DNA

Primers LCM15382 (5′ GCTTAACCCTAAAGCATTGG 3′) and H950g (5′ GTCTCGGATTTAGGGGTTTG 3′) (Abreu-Grobois et al. 2006) were used to amplify an 832-base-pair (bp) fragment at the 5′ end of the mitochondrial control region as described in Online Resource 2. We assigned haplotypes by comparing sequences to known reference haplotype libraries of 763 bp (Dutton et al. 1999; Dutton and Frey 2009). We standardized nomenclature of haplotypes based on these 763 bp alignments, assigning the Dc prefix to numerically sequential names based on the original 496 bp alignments (Dutton et al. 1999, 2007) with a sequential numerical suffix to indicate a variant resulting from polymorphism in the additional 267 bp region (LaCasella and Dutton 2008). We constructed statistical parsimony haplotype networks (Templeton et al. 1992, Posada and Crandall 2001) to depict patterns of genetic variation among the haplotypes by the median-joining (MJ) method (Bandelt and Forster 1999) using Network ver. 4.6.1.0 (http://www.fluxus-engineering.com).

We calculated haplotype (h) and nucleotide (π) diversity for each population using Arlequin v 3.5.1.2 (Excoffier and Lischer 2010). We tested for population structure by conducting analysis of molecular variance (AMOVA) (Excoffier et al. 1992), pairwise F_{ST} comparisons, and pairwise exact tests of population differentiation with Arlequin. Significance values for AMOVA were obtained from 10,000 permutations. Exact tests of population differentiation were conducted with 100,000 permutations and 10,000 dememorization steps (Raymond and Rousset 1995).

Nuclear DNA

We examined extracted DNA samples from nesting female leatherbacks using 17 polymorphic marine turtle microsatellite loci. Details of the reaction schemes for the 17 microsatellite primers may be found as follows: LB99, 14-5, LB110, LB128, LB141, LB142, LB145, LB143, LB133, LB123, LB125, LB157, LB158 (Roden and Dutton 2011); D1 and C102 (Dutton and Frey 2009); and N32 (Dutton 1995). One additional primer (D107; Dutton unpublished) was used with the following reaction scheme: initial denaturation for 5 min at 94 °C, followed by 35 cycles of 40 s at 94 °C (denature), 40 s at 58 °C (annealing) and 40 s at 72 °C (extension) and then a final extension for 5 min at 72 °C. All PCR products were checked for amplification using 2 % agarose gels with ethidium bromide staining. Microsatellite alleles were separated by electrophoresis on a Genetic Analyzer (ABI 3100, ABI 3130 or ABI Prism 3730) (Applied Biosystems, Foster City, CA, USA) using ROX 500 fluorescent size standard. We scored fragments using Genescan 3.1, Genotyper 2.0, or GeneMapper 4.0 software (Applied Biosystems, Foster City, CA, USA). We ran each PCR reaction and genotyping plate with positive and negative controls to ensure high genotyping quality and contamination-free reactions.

Microsatellite loci were screened for linkage disequilibrium and null alleles according to Roden and Dutton (2011). We tested for deviations from Hardy–Weinberg (HW) equilibrium via Markov chain permutation (Guo and Thompson 1992) using Genepop 4.1 (Raymond and Rousset 1995). In recent years several new statistics have been developed as an alternative to F_{ST} for nuclear data due to concern over biases as a measure of population structure, particularly when comparing results across different species or across different markers (Jost 2008; Meirmans and Hedrick 2011), although a general consensus has yet to be reached on which is the most appropriate and continued use of F_{ST} remains useful for within-species studies, such as ours (Whitlock 2011). We used several metrics to test for differentiation between rookeries; calculations of χ^2, F_{ST} (Weir and Cockerham 1984), and F'_{ST} (Meirmans and Hedrick 2011) were performed using custom scripts coded in R Development Core Team (2011, Archer unpublished). F'_{ST} corrects for differences between within-population diversity by standardizing F_{ST} values to the maximum diversity observed for each population. For all analyses, 10,000 permutations were used to calculate p values. In order to test for concordance between mtDNA and microsatellite patterns, we ran a Mantel correlation test in Arlequin using the F_{ST} matrices.

Additionally, we used the program POWSIM v.4.0 (Ryman and Palm 2006) to evaluate the statistical power of the mtDNA marker and the microsatellite markers to detect genetic differentiation at various levels of F_{ST}. This analysis simulates sampling from a specified number of populations that have reached pre-defined levels of divergence and estimates the probability of false negatives for population differentiation at the expected degree of divergence (Ryman et al. 2006). We simulated population drift to F_{ST} levels of 0.001, 0.0025, 0.005, 0.01, 0.02 and 0.05 by using an effective population size (Ne) of 1,000 and varying the number of generations (t) accordingly (Ryman et al. 2006). We determined the statistical power of our 17 microsatellite loci to detect differentiation among eight populations based on sample sizes of 50, 100, 200, 300, 400 and 600 and compared them with the POWSIM results for our mtDNA marker, adjusted for organelle (mtDNA) data (Larsson et al. 2009). Power is expressed as the proportion of significant outcomes (1,000 replicates, rejecting the null hypothesis (H_o) of no allele frequency difference, or $F_{ST} = 0$ at $p < 0.05$).

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Results

Mitochondrial DNA

Based on sequence data from 1,059 specimens, we identified ten mtDNA haplotypes defined by ten variable sites, all of which consisted of transitions (Table 1; Fig. 2; GenBank accession numbers HM452343-HM452352). Although we generated sequences that are 832 bp long using the primers listed above, we did not observe variation outside a 763 bp region within this dataset. The most common haplotype (Dc1.1), was present in all rookeries, occurring in 80.9 % of the samples analyzed (Table 2). Two haplotypes (Dc1.3 and Dc1.4) represent new variants of the original haplotype "A" based on the shorter (496 bp) sequence alignments reported in Dutton et al. (1999). We found these two new Dc1 variants only in the African rookeries (2.9 %). The second-most-common haplotype was Dc3.1, which we found at a frequency of 7 % in all of the western Atlantic (Caribbean) populations and in Gabon. We found an additional variant of the 496 bp haplotype "C" (Dutton et al. 1999) Dc3.2 in 3.1 % of the samples (Table 2), primarily in the Caribbean populations. Dc2.1 is a unique haplotype that we detected only in the St. Croix rookery (2 %), while we detected haplotype Dc4.1 and a new haplotype Dc13.1 in the West African rookeries at frequencies of 0.3 and 3.4 %, respectively (Table 2). We identified a fifth new haplotype (Dc17.1) in the Florida rookery and a sixth new haplotype (Dc19.1) in Costa Rica (Table 2), both at low frequencies.

Haplotype diversities among nesting sites based on the 763 bp sequences ranged from $h = 0.112$ to 0.498 (Table 2). Nucleotide diversities within nesting populations ranged from $\pi = 0.0004$ to 0.0032 (Table 2). Both are similar to values reported in Dutton et al. (1999) and Vargas et al. (2008), which were based on the shorter sequences (496 bp). Results of the AMOVA indicated significant population substructuring ($p < 0.001$, Table 3). Pairwise comparisons indicated significant differentiation between all populations except between FLA and ACR and TRI and GUI ($p < 0.01$, Table 3). The parsimony network shows a star-shaped phylogroup of six closely related haplotypes clustered around one common widespread haplotype (Dc1.1; Fig. 2). A second phylogroup consists of three haplotypes, with Dc3.1 widespread and separated by five steps from Dc1.1 (Fig. 2).

Nuclear DNA

We analyzed genotypes from a total of 1,417 samples. Analysis of Hardy–Weinberg deviation showed that none of the loci deviated significantly ($p < 0.05$) from equilibrium across all populations. Two loci showed significant ($p < 0.05$) deviation from HW expectations in three of the nine populations, while three loci deviated in only one population each (data not shown). We found significant F_{ST} values across all population pairs ranging from 0.004 (GHA vs. GAB, $p < 0.05$) to 0.205 (GAB vs. GUI, $p < 0.001$) (Table 4). F'_{ST} values were from 0.008 (GHA vs. GAB, $p < 0.05$) to 0.386 (GAB vs. GUI, $p < 0.001$) (Table 4). The allele frequencies were significantly different ($p < 0.001$) between all rookeries, as indicated by the χ^2 tests (results not shown). The Mantel test indicated a lack of correlation between pairwise mtDNA F_{ST} and microsatellite F_{ST} ($r = 0.16$, $p = 0.24$).

Table 1 Variable sites defining ten haplotypes based on sequences (763 bp) of the control region of mtDNA in Atlantic leatherbacks

Haplotype	Variable sites									
Current study (based on Dutton et al. 1999)	134 114	199 179	212 192	213 193	243 263	292 272	616	674	678	687
Dc1.1 (HM452343)	G	A	G	C	G	A	A	C	T	T
Dc1.3 (HM452344)	.	.	.	.	.	.	.	.	.	C
Dc1.4 (HM452345)	.	.	.	.	.	.	.	T	.	.
Dc2.1 (HM452349)	.	.	.	T	.	.	.	.	.	.
Dc3.1 (HM452350)	A	G	A	.	.	G	G	.	.	.
Dc3.2 (HM452351)	A	G	A	.	.	G	G	.	C	.
Dc4.1 (HM452352)	.	G	A	.	.	G	G	.	.	.
Dc13.1 (HM452346)	.	.	.	.	A	.	.	.	.	.
Dc17.1 (HM452347)	.	G	.	.	.	.	.	.	.	.
Dc19.1 (HM452348)	A	.	.	.	.	.	.	.	.	.

Haplotype designations from Dutton et al. (1999) are shown with corresponding variable positions and the GenBank accession number is given for each haplotype

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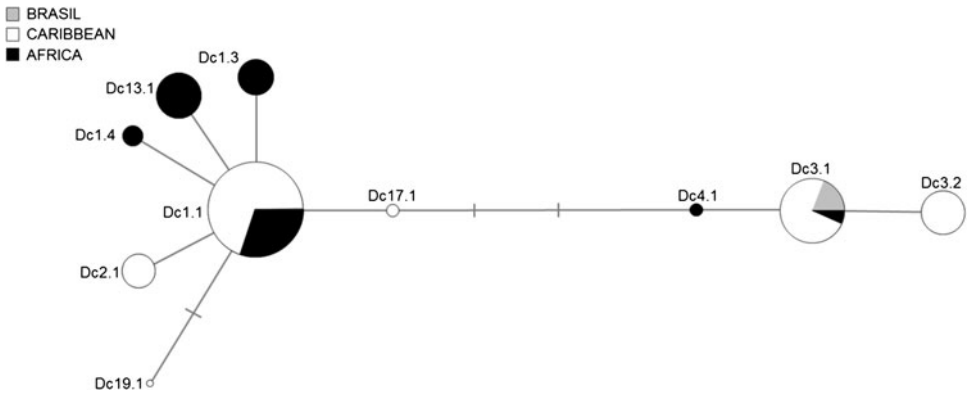

Fig. 2 The most parsimonious median-joining network of the 763 bp of the mtDNA control region for leatherback haplotypes in the Atlantic and SW Indian Ocean. The number of mutations between haplotypes is illustrated by dashes in connecting lines. The size of the *circles* is approximately proportional to haplotype frequency in the overall sample set. *Shadings* denote the regions where individual haplotypes were detected and the proportions of shared haplotypes that were distributed among rookeries in different regions

Table 2 Haplotype frequencies and diversity indices among Atlantic leatherback rookeries based on the mtDNA control region

Population		Haplotype frequency										Haplotype	Nucleotide
763 bp* 496 bp**	*n*	Dc1.1 A	Dc1.3 A	Dc1.4 A	Dc2.1 B	Dc3.1 C	Dc3.2 C	Dc4.1 D	Dc13.1 –	Dc17.1 –	Dc19.1 –	Diversity (h)	Diversity (π)
BRA	23	9				14						0.498 ± 0.053	0.0032 ± 0.0020
ACR	132	119				10	2				1	0.183 ± 0.044	0.0011 ± 0.0009
GUI	138	98				20	20					0.457 ± 0.044	0.0030 ± 0.0018
STX	123	98			21	4						0.338 ± 0.047	0.0008 ± 0.0007
TRI	87	65				11	11					0.415 ± 0.058	0.0027 ± 0.0017
FLA	222	209				10				3		0.112 ± 0.028	0.0006 ± 0.0006
GHA	61	47	11	1				1	1			0.379 ± 0.068	0.0006 ± 0.0006
GAB	232	178	12			5		2	35			0.387 ± 0.037	0.0008 ± 0.0007
SAF	41	34		7								0.298 ± 0.078	0.0004 ± 0.0004

Haplotype (*h*) and nucleotide diversities (π) were calculated using the 763 bp fragment. GenBank accession numbers are given for each haplotype and 1,059 turtle samples were analyzed

* Nomenclature based on 763 bp (current study) and ** Nomenclature based on 496 bp (Dutton et al. 1999)

Table 3 Pairwise F_{ST} values (above the diagonal; n/s = $p > 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$) and *p* values of exact tests of population differentiation (below the diagonal) among nine leatherback rookeries based on 763 bp sequence mtDNA haplotypes

	BRA	ACR	GUI	STX	TRI	FLA	GHA	GAB	SAF
BRA		0.539***	0.254***	0.412***	0.289***	0.676***	0.389***	0.383***	0.438***
ACR	<0.0001		0.077***	0.069***	0.058**	$0.005^{n/s}$	0.094***	0.064***	0.075**
GUI	<0.0001	<0.0001		0.074***	$-0.007^{n/s}$	0.144***	0.073***	0.068***	0.079**
STX	<0.0001	<0.0001	<0.0001		0.062***	0.111***	0.073***	0.064***	0.071**
TRI	<0.0001	0.0016	0.8334	<0.0001		0.127***	0.064***	0.056***	0.068***
FLA	<0.0001	0.0583	<0.0001	<0.0001	<0.0001		0.153***	0.097***	0.125***
GHA	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		0.034*	0.063*
GAB	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0001		0.061**
SAF	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0003	<0.0001	

Rookery abbreviations are given in Fig. 1

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Table 4 Pairwise F_{ST} (below diagonal) and F'_{ST} values (above diagonal) based on data from 17 microsatellite loci for nine leatherback rookeries in the Atlantic (see Fig. 1 for rookery abbreviations)

	BRA	ACR	GUI	STX	TRI	FLA	GHA	GAB	SAF
BRA		0.223*	0.223*	0.233*	0.211*	0.254*	0.229*	0.242*	0.193*
ACR	0.115*		0.107*	0.021*	0.061*	0.016*	0.319*	0.346*	0.301*
GUI	0.112*	0.055*		0.149*	0.011*	0.131*	0.356*	0.386*	0.364*
STX	0.120*	0.011*	0.077*		0.093*	0.030*	0.333*	0.360*	0.305*
TRI	0.105*	0.032*	0.006*	0.048*		0.081*	0.334*	0.363*	0.324*
FLA	0.136*	0.008*	0.069*	0.016*	0.043*		0.310*	0.337*	0.302*
GHA	0.119*	0.167*	0.184*	0.175*	0.172*	0.169*		0.008**	0.126*
GAB	0.131*	0.186*	0.205*	0.193*	0.193*	0.186*	0.004**		0.160*
SAF	0.091*	0.151*	0.177*	0.152*	0.155*	0.156*	0.062*	0.084*	

* Indicates significant $p < 0.001$, ** $p < 0.05$

Power analysis

Results of the POWSIM analysis showed that the mtDNA marker had low power in detecting weak differentiation, particularly at lower sample sizes (Fig. 3). The power to correctly detect structure with mtDNA with $F_{ST} < 0.005$ was less than 0.32 for a sample size of 50 and less than 0.66 with a sample size of 100. Power increased in general for mtDNA with larger sample sizes and was close to 1.0 for sample sizes ≥200 with $F_{ST} \geq 0.005$ but only reached 0.65 with a sample size of 400 with $F_{ST} \leq 0.001$ (Fig. 3). Our microsatellite array was able to reliably detect structure when F_{ST} was 0.0025 (power >0.99) for sample sizes ≥50 and when $F_{ST} = 0.001$ when sample sizes were ≥100. Power was lowest (0.61) when $F_{ST} = 0.001$ with a sample size of 50 (results not shown).

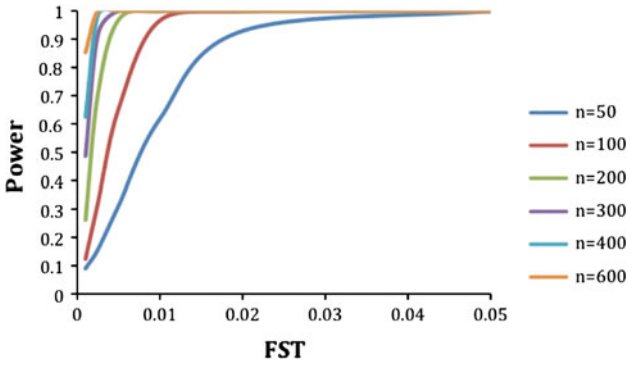

Fig. 3 POWSIM analysis results showing power to detect stock structure among Atlantic leatherback rookeries at different F_{ST} levels with different sample sizes using mtDNA control sequence variation. Power is expressed as the proportion of significant outcomes (1,000 replicates, rejecting the null hypothesis (H_o) of no allele frequency difference, or $F_{ST} = 0$ at $p < 0.05$)

Discussion

Stock structure

This study provides a comprehensive integration of nuclear and mtDNA data to address population stock structure in leatherbacks and our results demonstrate that breeding populations are more strongly differentiated than previously found with shorter (496 bp) mtDNA sequences (Dutton et al. 1999) and limited microsatellite data (Dutton 1995). Our mtDNA results allowed us to clearly identify seven MUs (Moritz 1994) for leatherbacks in the Atlantic, based on the significant level of differentiation both with mtDNA and microsatellites, and to further distinguish 2 DIPs based on microsatellites (discussed further below). For mtDNA, the additional variants of the common haplotype (Dc1) identified by the longer sequences now allow the South African rookery to be distinguished from the Caribbean rookeries which were indistinguishable based on the shorter (496 bp) sequences (Dutton et al. 1999). Furthermore, our study characterizes two of the major nesting populations of leatherbacks in western Africa (Gabon and Ghana) and identifies four new haplotypes that are observed only in African rookeries. An additional variant of Dc3 was found almost exclusively in the Caribbean rookeries (GUI, TRI, and ACR). These data now provide baseline information to assess the stock composition of foraging aggregations and identify natal origins of turtles caught in fisheries bycatch. In their study of leatherbacks caught in pelagic fisheries and strandings along the coast of Brazil, Vargas et al. (2008) described four haplotypes based on 711 bp sequences from leatherbacks that are equivalent to the haplotypes based on our longer 763 bp sequences (Dc1.3, Dc1.4, Dc4.1 and Dc13.1). Our results now solve the mystery of the origin of these haplotypes and confirm that leatherbacks of West African nesting stock origin migrate to foraging areas off the South American coast (see Vargas et al. 2008), which has recently

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been shown with flipper tagging and satellite telemetery as well (Billes et al. 2006; Witt et al. 2011).

There is however, a need for more extensive sampling of the numerous nesting sites throughout the Caribbean and West Africa to enable a full understanding of stock boundaries within these regions. For example, ACR and GUI are distinct stocks, but from tag returns (resightings of flipper-tagged turtles) on nesting beaches between Panama, Colombia, Venezuela and Guyana, it is possible that either a boundary or cline exists between the distinct stocks (Turtle Expert Working Group 2007). Most likely these countries, that are part of the Guyana Shield, comprise one regional stock made up of interconnected subpopulations with fuzzy boundaries that probably results from flexible natal homing i.e., turtles distributing nests up to >400 km apart between seasons (Troëng et al. 2004; Chacón-Chaverri and Eckert 2007) and up to 463 km between nests laid within the same nesting season (Stewart et al. personnel communication). The dynamic nesting beaches (Schultz 1979; Augustinus 2004) have undoubtedly also helped shape population structure along the Guyana Shield since beaches erode and accrete at timescales far shorter than the reproductive lifetime of leatherback turtles. A leatherback returning to nest may not encounter any suitable beaches in the vicinity where it originated. Our results show that the two extreme edges of the regional stock (ACR and GUI) are significantly different as indicated by both the microsatellite and mtDNA results (Tables 3, 4). A similar situation exists for loggerhead turtles (*Caretta caretta*) on the US East coast, where there are major nesting aggregations in Florida and Georgia, and low density nesting in between, with the population genetics characterized by a clinal shift in haplotype frequencies of two dominant haplotypes from north to south (Shamblin et al. 2011). Elsewhere in the Caribbean, St. Croix (USVI) should be considered as representative of a broader northern Caribbean genetic stock that probably includes Culebra Island, Vieques Island, Puerto Rico and the British Virgin Islands, based on resightings of flipper and passive integrated transponder tags (Turtle Expert Working Group 2007; Donna Dutton and Jeanne Garner personnel communication). Other Caribbean rookeries at St. Lucia, Dominican Republic, and Grenada have yet to be surveyed. In West Africa, the Gabonese (GAB) rookery sampled in our study is believed to be the largest in the world (Witt et al. 2011), however there are several other rookeries that should be surveyed to determine the level of regional sub-structuring. These include an important rookery on Bioko Island in Equatorial Guinea as well as smaller nesting populations in Ivory Coast and northern Gabon, as well as to the south in Congo and Angola.

Generally it had been thought that leatherbacks exhibited a considerably lower degree of site fidelity than other sea turtle species (Dutton et al. 1999), but based on data from both mtDNA and microsatellites, our findings indicate a higher degree of natal homing than previously reported. Our results also challenge the paradigm that has been espoused for sea turtles, which was that there was restricted gene flow of female lineages (mtDNA) maintained by natal homing, but that there was a tendency for homogenization of regional rookeries in terms of genomic DNA due to male-mediated gene flow (Karl et al. 1992). In contrast with previous findings by Dutton et al. (1999), who reported significant differentiation ($F_{ST} = 0.35$) between Trinidad (TRI) and Suriname (GUI), our more comprehensive present study indicates that TRI and GUI comprise one homogenous stock in terms of mtDNA. The earlier finding is likely due to sampling bias, as it was based on small sample sizes (TRI = 20; GUI = 27), and at the time these aggregations were considered one of the largest in the Atlantic (Girondot and Fretey 1996; Dutton et al. 1999). Dutton (1995) also found a lack of differentiation between these two populations based on three microsatellite loci and suggested that this pattern was evidence for male-mediated gene flow. This scenario is consistent among several studies that have used microsatellites (FitzSimmons et al. 1997; Roberts et al. 2004), however in most of these studies only a few loci were used providing a lower level of statistical power to detect differentiation. With the increased power of our more comprehensive microsatellite analysis, our finding of weak, but highly significant differentiation between TRI and GUI taken together with lack of mtDNA differentiation suggests that male mediated gene flow by itself is not responsible for the apparent connectivity. Interchange of nesting females, or via hatchlings recruiting as adult nesters to the other population are equally parsimonious explanations. A few (at least six) adult nesters tagged over a 10 year period in TRI have been observed nesting in subsequent years in GUI, and vice versa (Scott Eckert personnel communication), however it is unclear, given the large size of these populations, what impact this would have on genetic differentiation. Recently, a male leatherback was tracked by satellite telemetry from foraging areas in the northwest Atlantic to the same breeding area adjacent to the Trinidad nesting beaches 2 years in a row (James et al. 2005), supporting breeding fidelity of males.

Phylogeography

Our mtDNA parsimony network illuminates the demographic history of the leatherback in the Atlantic and expands upon what was known from the previous global study. Haplotype Dc4 was previously only identified in the Indo-Pacific and believed to be an ancestral haplotype that survived a global population contraction during Pleistocene glaciations (Haplotype D in Dutton et al. 1999). Our discovery of this haplotype in the West African rookeries (GHA and GAB) now suggests that post-Pleistocene

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recolonization of the Atlantic most likely occurred via the eastern Atlantic. Furthermore the central position of Dc3.1 in one of the two "local" Atlantic haplogroups present in throughout the Caribbean, as well as Brazil and Gabon suggests migration from both West Africa and the Caribbean to Brazil (Dutton et al. 1999).

The recent emergence of a growing leatherback population in Florida provides an opportunity to explore colonization and gene flow scenarios in leatherbacks. Leatherback populations have been increasing over the last decade in the northwestern Atlantic (Turtle Expert Working Group 2007), and in Florida nesting has increased 10.2 % per year over the past 30 years (Stewart et al. 2011). This pattern is very similar to the increase observed for St. Croix (Dutton et al. 2005). Our genetic results do not indicate any evidence of emigration from St. Croix to Florida. The mtDNA homogeneity we found between Costa Rica and Florida indicates that Costa Rica may be the source of the Florida population, and the high connectivity between these two rookeries is either due to ongoing recruitment of nesters born in Costa Rica to the growing Florida breeding population, or one or multiple recent colonization events from Costa Rica. Leatherbacks leaving Costa Rica may travel into the Gulf of Mexico and then further north to foraging grounds in Canada and the northeast US (Evans et al. 2007; Troëng et al. 2007), so it is conceivable that turtles may encounter suitable nesting habitat in Florida during this migration.

Conservation implications

Our findings do raise questions as to what level of differentiation between proximate rookeries, particularly when there is weak differentiation, warrants recognition of distinct populations. Even with the longer sequences now available, our POWSIM analysis showed that the mtDNA assay does not have the power to detect the weak differentiation that is reliably detected with our array of microsatellite markers. When haplotype frequencies are found to be similar between nesting populations, there is a danger of Type II error by failing to detect population differentiation because of insufficient resolution. This leads to lumping populations incorrectly and failing to identify the appropriate units to conserve (Taylor and Dizon 1999). Based on the mtDNA analysis, the pairwise F_{ST} for FLA and ACR, and for TRI and GUI are not significantly different from zero (Table 3). However, the microsatellite data analysis demonstrated weak differentiation ($F'_{ST} = 0.016$ and $F'_{ST} = 0.011$ respectively, Table 4), with highly significant p values (Table 4). Interestingly in West Africa, the Gabon and Ghana rookeries are relatively well differentiated based on mtDNA data ($F_{ST} = 0.03$), but weakly differentiated based on microsatellite data ($F_{ST} = 0.004$, $F'_{ST} = 0.008$, Table 4). The lack of concorndance between the mtDNA and microsatellite patterns in our study indicate that both male and female natal fidelity are not entirely responsible for shaping population structure. This discordance between markers has surprisingly been reported in marine fish that lack the confounding influence of sex-biased dispersal (DiBattista et al. 2012). While male-mediated gene flow might result in this pattern in our case, other factors could also produce higher F_{ST} statistics for mtDNA relative to nuclear data, and simulation studies have shown that higher mtDNA F_{ST} occur in populations that have recently diverged and have not yet reached equilibrium (Larsson et al. 2009). It is common for natural populations to have higher mtDNA F_{ST} relative to nuclear F_{ST} (see Karl et al. 2012). Possible explanations for the dissonance between markers in our study include (1) more rapid genetic drift occuring in mtDNA genes relative to nuclear genes precipitated by recent colonization, and (2) biased sex ratios resulting from temperature dependent sex determination (Mrosovsky and Godley 2010) amongst several others (see DiBattista et al. 2012). Regardless of the underlying factors, recognizing demographically independent nesting (female) populations is the appropriate management unit for conservation for sea turtles, since isolated rookeries will not be easily replaced once depleted.

Although mtDNA results indicate GUI and TRI comprise a single MU, the finding of weak microsatellite differentiation between these proximate populations indicates some level of demographic structuring that warrants recognition for GUI relative to TRI and FLA relative to ACR as DIPs (see Taylor et al. 2010). While there is no absolute level of measurable discreetness that applies to all situations, the detection of even weak genetic differentiation (e.g. $F_{ST} < 0.002$) in many cases signals sufficient demographic isolation for management of species of concern on relatively short (decades) ecological time scales (Taylor 1997; Waples and Gaggiotti 2006; Mesnick et al. 2011).

On a broader scale, Wallace et al. (2010) describe a framework for sea turtle conservation with nesting populations as fine-scale management units and regional locations connected by gene flow as regional management units (RMU), somewhat similar to DPSs. Our results fit the three RMUs they identify for the Atlantic, with relatively greater connectivity among Caribbean and African rookeries relative to Brazil and each other, corresponding to the Northwest, Southwest and Southeast Atlantic RMUs.

Finally, this work has fundamental implications for the management of leatherback turtles in the Atlantic basin, as we now have documented seven clearly distinct MUs identifiable with mtDNA data and nine DIPs identifiable with nuclear markers. This baseline of genetic stocks allows for the testing of many hypotheses about leatherback biology and conservation in the Atlantic, including

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but not limited to identification of origins of mixed stocks at foraging grounds, natal beach origins of turtles captured incidentally in fisheries and stranded on shorelines, and changes in population demography for the species. Additionally, the connectivity between some populations indicates that ongoing multilateral conservation programs should be maintained or expanded to allow for the sharing of information and the long-term protection of this transboundary species.

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